

REMARKS/ARGUMENTS

STATUS OF THE CLAIMS.

Claims 1, 4-9, 12, and 14-19 are pending in the application with claims 2, 3, 10, 11, and 13 being previously cancelled. Claims 1, 12, and 17 are amended herein to more clearly describe embodiments of the invention. These changes introduce no new matter and support is present in the application and claims as originally filed. The changes are made without prejudice and are not to be construed as abandonment of any previously claimed subject matter or agreement with any objection or rejection of record. Accordingly, entry of the Amendment is respectfully requested.

AMENDMENTS TO THE CLAIMS

Claims 1, 12, and 17 are amended herein. Support for such changes can be found throughout the application as filed.

The cited claims are merely amended to remove language from the currently claimed embodiments concerning changes in intracellular cAMP, cGMP, IP₃, and DAG and “wherein the functional effect is a change other than a change in Ca++ influx through a TC-ICS.” It will be appreciated that Applicants respectfully reserve the right to reinstate such claim coverage in this or other related applications. Because no new matter is added in any way by the changes, their entry is respectfully requested.

Further discussion of the amendments herein is presented below.

REJECTIONS TO THE CLAIMS

35 U.S.C. §112 First Paragraph

Written Description

Claims 1, 4-9, 12, and 14-17 were rejected in the Office Action as allegedly lacking adequate written description under U.S.C. §112, first paragraph. Applicants herein amend and traverse to the extant that the rejection remains after amendment.

In order to satisfy the written description requirement, M.P.E.P. §2163(I) states that “a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention.”

The Office Action contends that the cited claims lack adequate written description because “the limitation ‘wherein the functional effect is a change other than a change in Ca⁺⁺ influx through a TC-ICS; thereby identifying the compound that modulates taste signaling in taste cells’ is new matter that is wholly unsupported by the instant specification as filed.” Office Action at page 2. The Office Action also alleges that “[t]here is no disclosure in the specification as to whether a measured change in intracellular Ca⁺⁺ is...‘influenced’ by the modulation of a protein of the instant invention” and that “[t]he instant specification does not identify a specific mechanism through which the modulation of TC-ICS is supposed to effect intracellular Ca⁺⁺ in response to a test compound.” Office Action at page 3.

While Applicants believe the claims to have adequate written description, in order to further prosecution, Applicants herein amend claims 1, 12, and 17 (and hence their dependents) to remove the limitations concerning changes in intracellular cAMP, cGMP, IP₃, DAG, or Ca⁺⁺ as well as the limitation concerning changes “other than a change in Ca⁺⁺ influx through a TC-ICS.”

Because the language upon which the rejections were based has been removed, Applicants respectfully request that the rejections be withdrawn.

Enablement

Claims 1, 4-9, 12, and 14-17 were rejected under 35 U.S.C. § 112, first paragraph as allegedly failing to comply with the enablement requirement. Applicants respectfully traverse.

The Office Action contends that the “specification describes a protein identified as...a (TC-ICS)” but that [n]o functionality has been demonstrated for this protein.” The Office Action further alleges that “there is no evidence or sound scientific reasoning provided by the instant specification that TC-ICS is capable of functioning as a homomeric unit or of ‘modulating’ intracellular Ca^{2+} levels.” The Office Action also alleges that the specification does not “disclose, with specificity, precisely what effect the activation or inhibition of TC-ICS activity can be expected to have upon an individual” and that without such information those of skill in the art cannot “practice the claimed assay and employ the information obtained therefrom in a specific and substantial utility in currently available form.”

The Office Action summarizes its points by stating that the “specification leaves it to the artisan to make the substantial inventive contribution of determining if TC-ICS is capable of effecting intracellular Ca^{2+} levels at all, if the stimulation of TC-ICS elevates or suppresses intracellular Ca^{2+} levels, and of establishing a nexus between the activation of TC-ICS and the preceptor of a specific taste.”

Applicants respectfully disagree. The application, in fact, teaches not only functional effects of TC-ICS activity (e.g., changes in membrane potential), but also its role in taste signaling. The specification teaches: determination of TC-ICS activity (and determination of modulation of such); that TC-ICS is involved in taste signaling in taste cells; and that TC-ICS can be used to identify compounds that modulate taste signaling in taste cells.

In numerous locations, the specification teaches that TC-ICS activity and modulation can be determined through changes in functional effects, e.g., changes in intracellular ion concentration, changes in transmembrane ion flux, and changes in membrane potential. The specification as filed lays out numerous ways of detecting TC-ICS activity and modulation. For example, page 6, lines 3-15, page 8, lines 15-25, page 10, lines 24-31, page 11, lines 14-22, and pages 23-28 teach aspects of determination of TC-ICS activity. Furthermore, numerous additional citations are present in the specification and offer yet more guidance in determination of TC-ICS activity, e.g., via changes in membrane potential. The specification also gives examples of methods (such as dye localization, patch clamps, etc.) that can be used in the claimed assays (e.g., in determination of intracellular ion concentration, ion flux, changes in membrane potential) to measure any changes in functional effect that may result from a putative modulator.

It will be appreciated that the Office Action's focus on TC-ICS' influence on intracellular calcium concentration is moot because Applicants herein remove such limitation from the claims. *See above.*

Not only are the claimed ways of determining TC-ICS activity presented in the specification, but, those of skill in the art are extremely familiar with, and routinely perform such assays to measure/quantify functional effects of modulators on cells/cell membranes, e.g., by measuring ion flux, changes in membrane potential, etc. As evidence of such, the specification cites to multiple journal articles detailing protocols to guide those of skill in the art in such determination.

Additionally, as taught in the specification, TC-ICS is an ion channel (e.g., page 7, page 9, etc.). Thus, there is logical scientific reasoning (and, as shown below, evidentiary support) that TC-ICS activity and modulation of such can, at the least, be tracked through determination of changes in intracellular ion concentration, ion flux, and changes in membrane potential. As is well known in the art, ion channels such as TC-ICS typically act to allow ion flow through membranes which, in turn, alters net intracellular charge. The teachings of the specification of changes in membrane potential, etc. from TC-ICS activity, were verified in Zhang. *See below.*

The nexus between TC-ICS activity/modulation and taste signaling is also taught throughout the specification. Additionally, the isolation of TC-ICS from specific taste cells (e.g., page 4, lines 11-15, Example I, etc.) and the identification of TC-ICS as an ion channel in such cells (e.g., page 7, page 9, Example I, etc.), present logical support of the teachings in the specification of the connection between taste signaling and TC-ICS activity (e.g., page 4, lines 18+, page 23, etc.). Thus, there is more than reasonable correlation between the TC-ICS information and the uses taught.

Not only are the functional effects of TC-ICS and the connection between TC-ICS activity and taste signaling taught in the application, but such matters are explicitly verified by Zhang, *et al.*, in "Coding of Sweet, Bitter, and Umami Tastes: Different Receptor Cells Sharing Similar Signaling Pathways," 2003, Cell, 112:293-301. Zhang corroborates the teachings present in the application and clearly supports the measurement of changes in functional effects, such as changes in membrane potential, in showing TC-ICS activity and the nexus between TC-ICS activity and taste signaling as taught in the specification.

Zhang is explicit in its support of the teachings of the instant specification. For example, Zhang shows use of patch clamp techniques to determine change in membrane potential

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due to TC-ICS activity (labeled as TRPM5 activity in Zhang). *See, e.g.,* Figure 2 and “TRPM5 Is Activated by GPCR Signaling” on pages 294-295. Furthermore, the teachings of the specification concerning the nexus between TC-ICS activity and taste signaling are also supported by Zhang, *e.g.,* in Figure 4 which demonstrates that TC-ICS mutants do not show responses to particular taste stimuli and Figure 5 which shows that TC-ICS knock out animals lacked specific taste signaling (as measured by preference response).

Thus, the specification clearly has an established practical utility that was recognized and appreciated by the inventors at the time of the invention. The subsequent evidence as shown by Zhang verifies and supports the embodiments produced/taught in the specification. *See, e.g., Cooper v. Goldfarb*, 154 F.3d 1321, 47 USPQ2d 1896 (Fed. Cir. 1998) approving use of post-filing publications to verify utility.

Because the specification clearly taught and appreciated the determination of TC-ICS modulation (*e.g.*, via changes in intracellular ion concentration, changes in transmembrane ion flux, and changes in membrane potential) and the nexus between such modulation and taste signaling and because both of such points were verified and corroborated by subsequent evidence, the invention does have an established utility and the claims are fully enabled. Therefore Applicants respectfully request that the rejection be withdrawn.

35 U.S.C. §112 Second Paragraph

Indefiniteness

Claims 1, 4-9, 12, and 14-19 were rejected in the Office Action as allegedly indefinite under 35 U.S.C. §112, second paragraph. Applicants herein amend and respectfully traverse to the extent that the rejections are maintained after amendment.

The Office Action contends that the phrase “a change in intracellular Ca^{2+} ” is indefinite because it does not describe what is changing in regard to intracellular Ca^{++} (*e.g.*, concentration, localization, or isotopic composition). Furthermore, the Office Action states that such lack is also true for the limitations concerning “a change in intracellular cAMP, cGMP, IP_3 , or DAG.”

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As stated above, Applicants herein amend claims 1, 12, and 17 (and hence their dependents) to remove limitations concerning determination of functional effects wherein the effect is chosen from "a change in intracellular cAMP, cGMP, IP3 or DAG, and a change in intracellular Ca⁺⁺; and wherein the functional effect is a change other than a change in Ca⁺⁺ influx through a TC-ICS." Such changes thus clarify that the functional effects being determined in the current embodiments are chosen from: a change in intracellular ion concentration, a change in transmembrane ion flux, and a change in membrane potential.

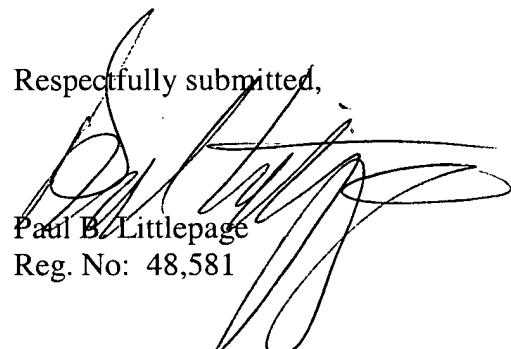
Because the language of upon which the rejection was based has been removed, Applicants respectfully request that the rejection be withdrawn.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested. In the event that substantive matters are felt to remain, the Examiner is invited to telephone the undersigned at (510) 769-3507.

QUINE INTELLECTUAL PROPERTY LAW
GROUP, P.C.
P.O. BOX 458
Alameda, CA 94501
Tel: 510 337-7871
Fax: 510 337-7877

Respectfully submitted,


Paul B. Littlepage
Reg. No: 48,581

Coding of Sweet, Bitter, and Umami Tastes: Different Receptor Cells Sharing Similar Signaling Pathways

Yifeng Zhang,¹ Mark A. Hoon,^{2,3}
Jayaram Chandrashekar,^{1,3} Ken L. Mueller,^{1,3}
Boaz Cook,¹ Dianqing Wu,⁴

Charles S. Zuker,^{1,*} and Nicholas J.P. Ryba²
¹Howard Hughes Medical Institute

Departments of Biology and Neurosciences
University of California, San Diego
La Jolla, California, 92093

²National Institute of Dental and Craniofacial
Research
National Institutes of Health, DHHS
Bethesda, Maryland 20892

Summary

Mammals can taste a wide repertoire of chemosensory stimuli. Two unrelated families of receptors (T1Rs and T2Rs) mediate responses to sweet, amino acids, and bitter compounds. Here, we demonstrate that knockouts of TRPM5, a taste TRP ion channel, or PLC β 2, a phospholipase C selectively expressed in taste tissue, abolish sweet, amino acid, and bitter taste reception, but do not impact sour or salty tastes. Therefore, despite relying on different receptors, sweet, amino acid, and bitter transduction converge on common signaling molecules. Using PLC β 2 taste-blind animals, we then examined a fundamental question in taste perception: how taste modalities are encoded at the cellular level. Mice engineered to rescue PLC β 2 function exclusively in bitter-receptor expressing cells respond normally to bitter tastants but do not taste sweet or amino acid stimuli. Thus, bitter is encoded independently of sweet and amino acids, and taste receptor cells are not broadly tuned across these modalities.

Introduction

The sense of taste is responsible for detecting and distinguishing between sweet, bitter, sour, salty, and amino acid (umami) stimuli. This discriminatory power provides animals with critical sensory input: sweet and amino acid receptors allow recognition of nutritionally rich food sources, while bitter receptors elicit aversive responses to noxious and toxic stimuli. Recently, we utilized cell-based assays, genetics, bioinformatics, and expression studies to functionally identify mammalian taste receptors. Sweet and amino acid tastes (umami) are mediated by a family of three GPCRs (the T1Rs) that combine to generate at least two heteromeric receptors: T1R2 and T1R3 associate to function as a broadly tuned sweet receptor (Nelson et al., 2001; Li et al., 2002), while T1R1 and T1R3 form a universal L-amino acid sensor (Nelson

et al., 2002). Bitter tastants are detected by members of an unrelated family of ~30 different GPCRs, the T2Rs (Adler et al., 2000; Chandrashekar et al., 2000; Matsunami et al., 2000). Most T2Rs are co-expressed in the same subset of taste receptor cells of the tongue and palate epithelium (Adler et al., 2000), suggesting that these cells are capable of responding to a broad array of bitter compounds. T2Rs may also function as heteromeric receptors to accommodate the great chemical diversity of bitter tastants.

Mammalian taste receptor cells are small neuroepithelial cells, tightly packed into taste buds, that are distributed in distinct regions of the tongue and palate epithelium. Each taste bud, depending on the species contains 50–150 cells, including precursor cells, support cells, and taste receptor cells (Lindemann, 1996). Receptor cells are innervated by afferent nerve endings that transmit information to the taste centers of the cortex through synapses in the brain stem and thalamus. How are the various taste modalities encoded at the periphery? In the simplest model, specific taste qualities could be independently determined by different cells expressing different receptors. Recently, we showed that T1Rs and T2Rs are expressed in nonoverlapping populations of receptor cells within individual taste buds of the tongue and palate epithelium (Hoon et al., 1999; Adler et al., 2000; Nelson et al., 2001). We interpreted these results to imply that sweet, amino acid, and bitter taste modalities are encoded separately by the activation of distinct cell types. This contrasts current models of taste coding at the periphery, where individual taste cells have been proposed to respond to multiple taste qualities, including some thought to respond to sweet, amino acid, and bitter tastants (Gilbertson et al., 2001; Caicedo et al., 2002).

Signaling pathways downstream of taste receptors have also been the subject of intense speculation. There is evidence that T2R bitter receptors are co-expressed with gustducin, and T2Rs can activate this G protein in vitro (Adler et al., 2000; Chandrashekar et al., 2000). However, a knockout of gustducin still retains substantial sensitivity to bitter tastants in physiological and behavioral assays (Wong et al., 1996; He et al., 2002). The involvement of other components implicated in taste signaling is equally unclear. For example, even though natural and artificial sweeteners activate the same receptor (T1R2+3, Nelson et al., 2001; Li et al., 2002), they have been proposed to stimulate different signaling pathways resulting in the production of multiple second messengers (Bernhardt et al., 1996; Cummings et al., 1996). Sugars are believed to elevate cAMP while artificial sweeteners are proposed to induce production of InsP₃ (Gilbertson et al., 2000; Smith and Margolskee, 2001; Margolskee, 2002). Furthermore, cAMP has been proposed to activate taste receptor cells either by opening a cyclic nucleotide-gated channel (Misaka et al., 1997) or by activating protein kinase A to phosphorylate K⁺-channels (Cummings et al., 1996; Margolskee, 2002). Bitter taste signaling is believed to be no less complex and to also involve both cyclic nucleotides (by activation

*Correspondence: charles@flyeye.ucsd.edu

³These authors contributed equally to this work.

⁴Present address: University of Connecticut, UCHC, Farmington, Connecticut 06030.

of cyclic nucleotide phosphodiesterases [Ruiz-Avila et al., 1995] and even a cyclic nucleotide-suppressible conductance [Kolesnikov and Margolskee, 1995]) and InsP_3 (via calcium release, reviewed in Kinnamon, 2000; Smith and Margolskee, 2001; Margolskee, 2002). This proposed complexity contrasts sharply with the demonstrated simplicity of the signaling pathways in other senses. For instance, vision and olfaction each use a single signaling pathway. Importantly, in the case of olfaction, many hundreds of distinct receptors share an identical transduction cascade (Brunet et al., 1996). Similarly, two large unrelated families of pheromone receptors most likely require a single specific TRP-ion channel for signaling in the mammalian vomeronasal system (Stowers et al., 2002; Leybold et al., 2002). These examples illustrate that a single pathway can accommodate a wide range of receptor diversity. Indeed, we now provide evidence that sweet, amino acid, and bitter taste receptor cells use a common signaling pathway for the generation of a taste response, despite using different receptor systems. We examined knockouts of two signaling molecules selectively expressed in sweet, amino acid, and bitter taste receptor cells and show that these three modalities require a common TRP ion channel, TRPM5, and phospholipase C, PLC β 2. We also used cell-based assays to demonstrate that TRPM5 is activated by GPCR stimulation, but is not gated by Ca^{2+} , InsP_3 , or release from internal stores.

A long-standing controversy in taste coding relates to whether receptor cells mediate responses to specific or to multiple taste modalities. By selectively rescuing PLC function in bitter receptor-expressing cells of PLC knockout animals, we demonstrate that taste receptor cells are not broadly tuned across all taste modalities.

Results and Discussion

An Ion Channel Common to Sweet, Amino Acid, and Bitter Taste Receptor Cells

Perez et al. (2002) reported the isolation of a TRP gene, TRPM5, expressed in taste cells of the tongue. Using a subtractive screening strategy, we also identified TRPM5 and found that it was expressed in taste receptor cells throughout the oral cavity, including tongue and palate (see Experimental Procedures). TRPs are a diverse family of cation channels found in both vertebrates and invertebrates and implicated in calcium signaling, pain transduction, thermosensation, mechanotransduction, chemosensory signaling, and vision (e.g., see Clapham et al., 2001; Minke, 2001; Minke and Cook, 2002 for reviews). We reasoned that if TRP has a specific role in taste it should be expressed in selective subsets of taste receptor cells. Recently, sweet (T1R2+3, Nelson et al., 2001), amino acid (umami, T1R1+3, Nelson et al., 2002; Li et al., 2002), and bitter (T2Rs) taste receptors (Adler et al., 2000; Chandrashekar et al., 2000; Matsunami et al., 2000) were identified and shown to be expressed in mostly nonoverlapping subsets of taste receptor cells of the tongue and palate epithelium (Adler et al., 2000; Nelson et al., 2001). To determine which cells express TRPM5, we performed double-labeling experiments with T1Rs, T2Rs, and TRPM5 using two-color fluorescent detection. Our results showed that TRPM5 is ex-

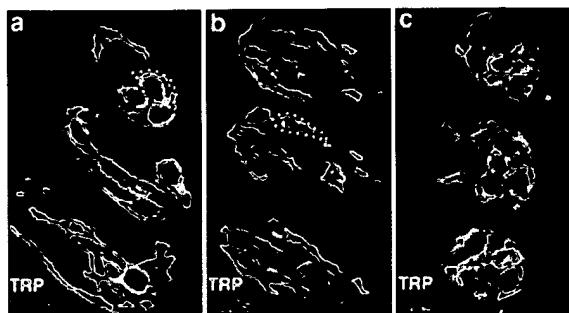


Figure 1. TRPM5 Is Co-Expressed with T1R- and T2R Taste Receptors

Double-label fluorescent immunohistochemistry (TRPM5) and *in situ* hybridization (T1Rs, T2Rs, and PLC β 2) were used to directly examine the overlap in cellular expression of taste receptors, TRPM5, and PLC β 2.

(A) Circumvallate taste buds illustrating co-expression of T1Rs (a mixture of T1R2 and T1R3, red) and TRPM5 (green). Approximately 50% of taste receptor cells express TRPM5; all cells expressing T1Rs also express TRPM5 and account for half of the TRPM5-positive cells.

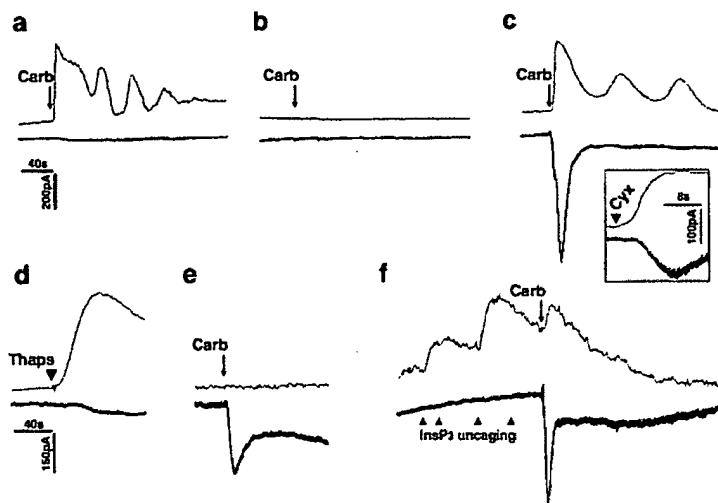
(B) Co-expression of T2Rs (a mixture of 25 T2Rs, red) and TRPM5 (green). Every T2R positive cell expresses TRPM5 and account for a further 40% of the TRPM5-positive cells. Thus, every T1R and T2R positive cell expresses TRPM5 and together account for ~90% of the TRPM5 cells. Dotted lines in (A) and (B) show the outline of a double-positive cell.

(C) Nearly all TRPM5-positive cells (green) co-express PLC β 2 (red). TRPM5 is not expressed anywhere else in the tongue, including taste fibers.

pressed in approximately 50% of the taste receptor cells, and that every T1R- and T2R-positive cell in circumvallate, foliate, fungiform, and palate taste buds co-expresses TRPM5 (Figure 1 and data not shown). Thus, TRPM5 is a taste-specific channel common to sweet, amino acid, and bitter-responding cells.

TRPM5 Is Activated by GPCR Signaling

If TRPM5 mediates taste responses in sweet, amino acid, and bitter receptor cells, we anticipated TRPM5 to be activated by signaling events downstream of GPCRs. Because many TRPs are known to be gated by events downstream of PLC β (Minke, 2001), we assayed TRPM5 function in cells expressing a G protein that couples a wide range of GPCRs, including taste and olfactory receptors, to PLC (Offermanns and Simon, 1995; Krautwurst et al., 1998; Chandrashekar et al., 2000). We transfected TRPM5 into HEK cells expressing G α 15 and stimulated either endogenous muscarinic receptors with carbachol or a cotransfected taste receptor (mouse T2R5) with cycloheximide (Chandrashekar et al., 2000). TRPM5 activity was monitored by patch clamp analysis and PLC activation by measuring changes in $[\text{Ca}^{2+}]_i$. Figure 2 shows that GPCR stimulation of cells co-expressing G α 15 and TRPM5 triggered both $[\text{Ca}^{2+}]_i$ mobilization and a robust TRPM5-dependent current. In contrast, cells expressing G α 15 but lacking TRPM5 produce strong $[\text{Ca}^{2+}]_i$ mobilization, indicative of activation of PLC, but no change in membrane conductance. HEK cells transfected with TRPM5 alone show neither $[\text{Ca}^{2+}]_i$ responses, nor carbachol (or cycloheximide) stimulated



(D-F) examine the roles of internal stores, $[Ca^{2+}]_i$ and $InsP_3$, and in the activation of TRPM5.

(D) Application of thapsigargin (Thaps) to cells expressing $G\alpha 15$ and TRPM5 releases $[Ca^{2+}]_i$ from internal stores, but fails to activate TRPM5. (E) Conversely, intracellular application of BAPTA abolishes $[Ca^{2+}]_i$ increases, but fails to prevent the carbachol-induced activation of TRPM5. (F) Finally, intracellular release of $InsP_3$ by UV-mediated uncaging of caged $InsP_3$ (arrowheads) results in increases in $[Ca^{2+}]_i$ but no activity from TRPM5. Note that subsequent application of carbachol triggers TRPM5 activation.

conductances. Thus, we conclude that TRPM5 encodes a functional channel activated by GPCR signaling.

Because a number of TRP channels have been proposed to be directly gated by Ca^{2+} or by emptying of internal stores (Clapham et al., 2001; Minke and Cook, 2002), we examined the role of Ca^{2+} and internal stores in the activation of TRPM5. Our strategy was to deplete the internal stores of TRPM5 transfected cells by application of thapsigargin and to manipulate intracellular calcium by release of caged $InsP_3$, caged Ca^{2+} , and treatment with BAPTA. As expected, $[Ca^{2+}]_i$ levels increased in response to thapsigargin (Figure 2D) and after UV-uncaging of caged $InsP_3$ (Figure 2F) or caged Ca^{2+} (data not shown). However, none of these treatments activated the TRPM5 ion channel. Conversely, application of BAPTA through the patch pipette blocked the carbachol-induced increase in $[Ca^{2+}]_i$, but had no effect on the generation of the TRPM5 current (Figure 2E). Together, these results demonstrate that TRPM5 can be activated by GPCR-signaling, but is not activated by calcium, $InsP_3$, or thapsigargin-mediated depletion of internal stores. Similar results have been obtained for the *Drosophila* photoreceptor TRP channels, the mammalian VNO TRP channel, and a large number of "orphan TRPs" (Clapham et al., 2001; Minke, 2001; Minke and Cook, 2002, but see also Montell et al., 2002).

TRPM5 Is Required for Sweet, Bitter, and Amino Acid Taste Signaling

To define the role of TRPM5 in taste, we generated knockout mice that lack a functional TRPM5 protein. Our strategy involved deleting exons 15 to 19 which encode the first five transmembrane domains and pore region of the channel. Figure 3 shows antibody labeling experiments demonstrating a complete lack of TRPM5 staining in homozygous KO animals. In order to ensure that loss of TRPM5 did not affect the viability or integrity

Figure 2. TRPM5 Encodes a Functional Ion Channel Activated by GPCR Signaling

Whole-cell patch clamp analysis (black traces) was used to measure TRPM5 activity in transfected HEK293 cells. Changes in $[Ca^{2+}]_i$ were simultaneously monitored using Fluo-3 (gray traces).

(A) Carbachol stimulation of cells expressing $G\alpha 15$ lead to robust increases in $[Ca^{2+}]_i$, but no significant changes in membrane conductance.

(B) Cells transfected with TRPM5 alone show neither channel activity nor $[Ca^{2+}]_i$ responses to carbachol. However, (C) when $G\alpha 15$ and TRPM5 are co-expressed, GPCR stimulation elicits both a robust $[Ca^{2+}]_i$ response and a large transient increase in membrane conductance. Similar results are obtained in cells co-expressing $G\alpha 15$ and TRPM5 with the mouse T2R5 bitter taste receptor and stimulated with cycloheximide (inset). Thus, TRPM5 encodes an ion-channel activated by GPCR stimulation.

of taste cells, we also compared the expression of T1Rs, T2Rs, and gustducin in control and KO animals; no significant differences were observed in the number or distribution of T1R-, T2R-, and gustducin-positive cells between wild-type and KO taste tissue (data not shown).

To examine the taste responses of the genetically modified mice, we recorded tastant-induced action potentials from the two major nerves innervating taste receptor cells of the tongue (chorda tympani at the front and glossopharyngeal at the back). This physiological

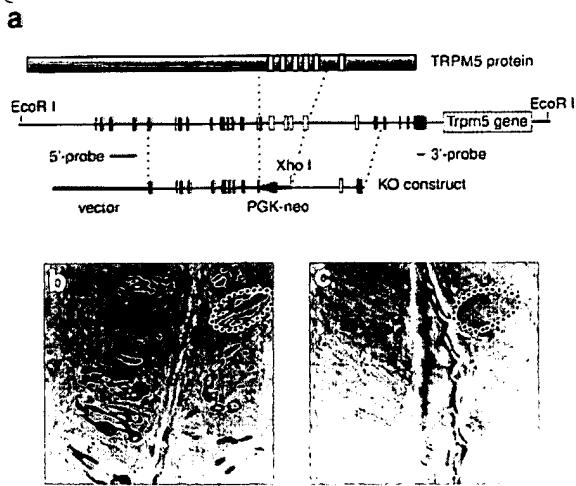
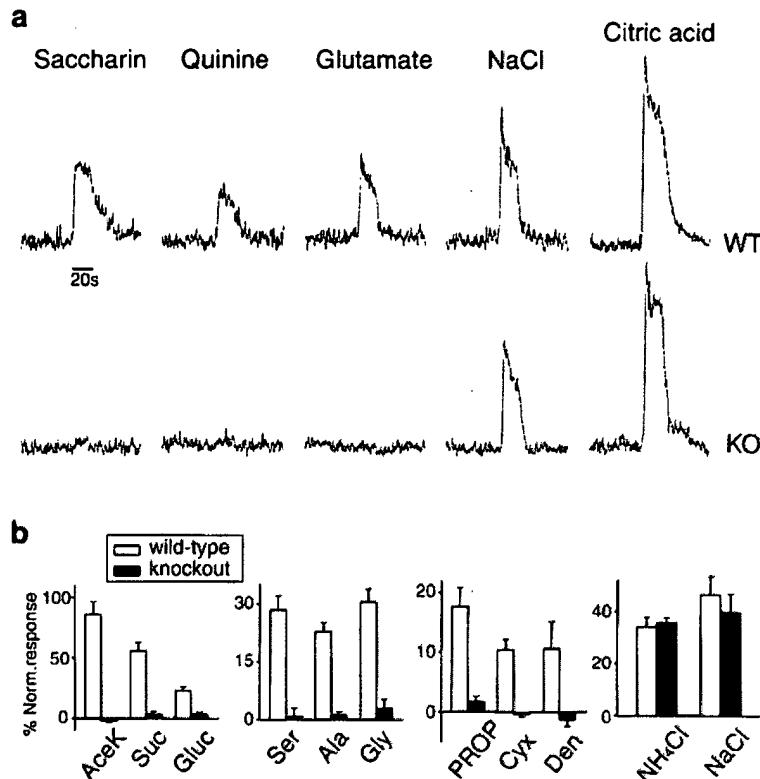


Figure 3. Targeted KO of TRPM5

(A) Schematic drawing showing the structure of the TRPM5 gene and the strategy for generating knockout animals. The targeting construct (lower diagram) deleted five of the six predicted transmembrane helices of TRPM5 (upper diagram). Immunohistochemistry staining demonstrates (B) robust expression of TRPM5 in taste buds of wild-type animals, but (C) complete absence in knockout animals.



assay monitors the coupling between taste receptor cells and afferent nerves and serves as a powerful indicator of the activity of the taste system at the periphery. In control mice, a variety of sweet, amino acid, bitter, sour, and salty stimuli elicit robust nerve responses (Figure 4). In contrast, responses to sweet, amino acid, and bitter stimuli are essentially abolished in the knockout animals (Figure 4). Indeed, we tested a panel of 9 sweet, 10 amino acid, and 6 bitter tastants, and in all cases failed to elicit a significant response. Notably, TRPM5 KO animals retain normal responses to salty or sour stimuli. A strong prediction of these physiological results is that TRPM5 KO mice should have severely compromised behavioral responses to sweet, bitter, and amino acid tastants, but normal salty and sour taste. Therefore, we examined taste behavior by measuring taste choices in two-bottle intake preference assays or by directly counting immediate licking responses in a 16-stimulus channel gustometer (see Experimental Procedures). As shown in Figure 5, the ability of the KO animals to taste sweet, bitter, or amino acid stimuli is completely abolished. However, both the KO and wild-type animals were equally sensitive to salty or sour stimuli. Taken together, these results demonstrate that TRPM5 is an obligatory component of sweet, amino acid and bitter taste reception and suggest that TRPM5-expressing cells are responsible for mediating all sweet, amino acid, and bitter responses. These data also illustrate two important points about salty and sour taste. First, salty and sour operate through distinct signaling pathways independent of TRPM5, and second, a functioning sweet, bitter, or amino acid modality is not required for salt and sour reception or perception.

Figure 4. TRPM5 Mutants Do Not Respond to Sweet, Amino Acid, or Bitter Stimuli

(A) Wild-type mice (WT) show robust neural responses to sweet (30 mM saccharin), bitter (1 mM quinine), amino acid (30 mM glutamate), salty (100 mM NaCl), and sour tastants (100 mM citric acid). In contrast, TRPM5 knockout animals (KO) show no responses to sweet, amino acid, or bitter stimuli, but display normal responses to salt and acid stimulation.

(B) Integrated neural responses, such as those shown in (A), were normalized to the response elicited by 100 mM citric acid. White bars, wild-type mice; red bars, TRPM5 knockout animals. The values are means \pm SEM ($n = 5$). Sweet tastants are: 60 mM acesulfame K (AceK), 300 mM sucrose (Suc), 300 mM glucose (Gluc); amino acids: 30 mM serine and alanine, 100 mM glycine; bitter: 10 mM 6-n-propyl thiouracil (PROP), 1 mM cycloheximide (CyX), 10 mM denatonium benzoate (Den); salts are 100 mM each. The data represent chorda tympani responses; similar phenotypes were obtained from glossopharyngeal nerve recordings (see Experimental Procedures for details).

PLC β 2 Is Required for Sweet, Amino Acid, and Bitter Taste

If signal transduction pathways in sweet, amino acid, and bitter receptor cells converge on a common TRPM5 channel, we hypothesized that additional components should be shared between these modalities. Recently, PLC β 2, one of the four PLC β isoforms, was shown to be expressed in taste receptor cells (Rossler et al., 1998). To examine its expression in detail, we performed double-labeling experiments with T1Rs, T2Rs, and TRPM5. Figure 1C demonstrates that PLC β 2 and TRPM5 have virtually overlapping patterns of expression in sweet, amino acid, and bitter receptor expressing cells (see also Miyoshi et al., 2001; Perez et al., 2002).

We envisaged that if TRPM5 is activated via PLC in vivo, then a PLC β 2 knockout should recapitulate the TRPM5 mutant phenotype and impair sweet, amino acid, and bitter tastes. PLC β 2 KO animals are viable, show no apparent phenotype at the systemic or cellular level (Jiang et al., 1997), and display no anomalies in the expression or distribution of T1Rs, T2Rs, gustducin, and TRPM5 (data not shown). To assay for taste phenotypes, PLC mutants were analyzed by electrophysiology and behavior. Figure 6 shows electrophysiological recordings of tastant-induced nerve activity to a panel of sweet, amino acid, bitter, salty, and sour compounds. Like TRPM5, PLC β 2 mutants have a selective and complete loss of sweet, amino acid, and bitter responses, but retain normal salty and sour responses. These results validate PLC β 2 as a key effector of mammalian sweet, amino acid, and bitter taste, and substantiate the proposal that PLC activation modulates TRPM5 function. Interestingly, when we assayed PLC β 2 mu-

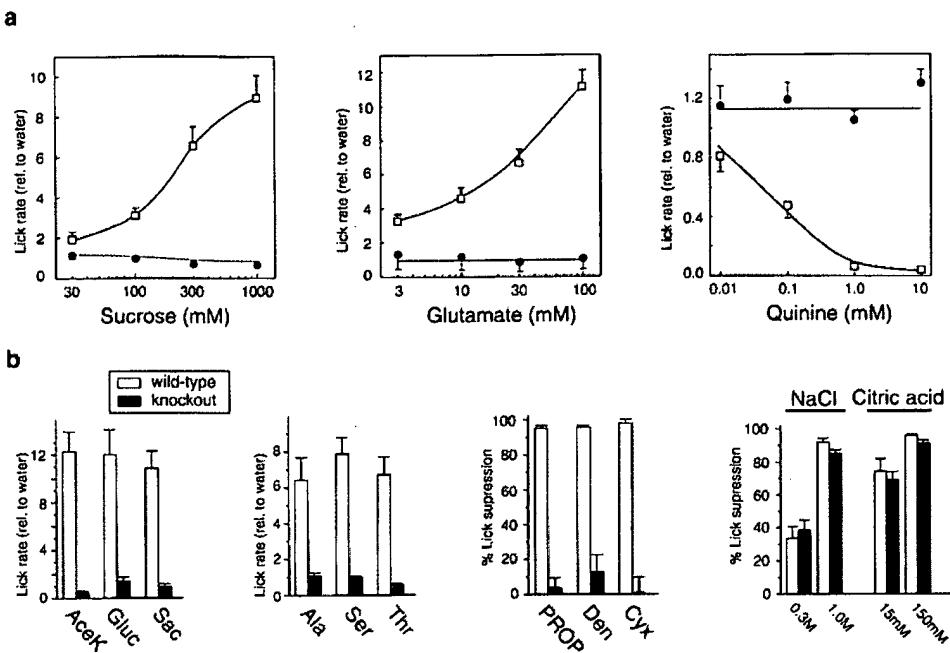


Figure 5. TRPM5 Knockouts Do Not Taste Sweet, Amino Acid, or Bitter Compounds

Taste preferences of wild-type (open squares, white bars) and TRPM5 knockout animals (red circles, red bars) were measured relative to water using a brief access taste test (Glendinning et al., 2002). Knockout of TRPM5 results in complete loss of the ability of animals to distinguish sweet (sucrose), amino acid (glutamate) and bitter tastants (quinine) from water over a wide concentration range (A). Similar results (B) were obtained for many other sweet, amino acid, and bitter tastants. In contrast, wild-type and knockout animals were equivalent in their ability to taste salty and sour stimuli. Sweet tastants: 32 mM acesulfame K, 2.5 M glucose, 32 mM saccharin; amino acids: 1 M alanine and serine, 300 mM threonine; bitter: 10 mM PROP and denatonium, 100 μ M cycloheximide. The values are means \pm SEM ($n = 8$). Similar results were obtained using a standard two bottle preference assay (data not shown).

tants by behavioral taste tests and compared them to TRPM5 knockout mice, PLC β 2 knockout may retain a small residual attraction to very high concentrations of sweet tastants (see Figure 7). This trace behavioral response could be due to functional redundancy by additional PLC isoforms in sweet cells.

Taste Receptor Cells Are Not Broadly Tuned across All Modalities

The availability of animals devoid of sweet, amino acid, and bitter taste provided a unique opportunity to functionally investigate taste coding. Recently, we characterized sweet, amino acid, and bitter receptors and showed they are expressed in largely nonoverlapping populations of taste cells. These results led us to propose that these three sensory modalities are encoded by activation of different subsets of cells (Nelson et al., 2001). This contrasts current models of taste coding at the periphery which propose that sweet, amino acid, and bitter perception are mediated by taste receptor cells broadly tuned across all modalities (Gilbertson et al., 2001; Caicedo et al., 2002). In such a scenario, one would expect receptors for multiple modalities to co-exist in the same cells. Thus, either there are additional yet undiscovered receptors in these cells, or the sensitivity of our expression studies does not accurately reflect the functional distribution of receptors. In order to experimentally address receptor cell tuning *in vivo*, we generated genetically modified mice in which PLC func-

tion was restored only in bitter-receptor expressing cells, and then assayed for taste responses across all modalities both electrophysiologically and behaviorally. If taste receptor cells are indeed broadly tuned to sweet, amino acid, and bitter tastes, then restoring taste function in a subset of cells, in this case T2R-expressing cells, should restore taste to multiple modalities. In contrast, if T2R-expressing cells are only tuned to bitter reception, as our receptor expression studies suggested (Hoon et al., 1999; Adler et al., 2000; Nelson et al., 2001), then restoring PLC activity in these cells should rescue bitter but not sweet or amino acid taste.

A 3.6 kb cDNA clone containing a wild-type PLC β 2 sequence was placed downstream of a promoter selectively expressed in bitter receptor cells (our unpublished data). In order to follow the presence and expression of the PLC β 2 transgene, we replaced its 3'-UTR and polyadenylation signal with that of bovine growth hormone. Our goal was to produce progeny that were homozygous for the PLC knockout allele, but carried the wild-type PLC transgene. Indeed, a PLC β 2 transgene fully rescues the bitter taste defect of PLC knockout animals, both physiologically (Figure 6) and behaviorally (Figure 7). In contrast, responses to sweet and amino acid tastants remain severely impaired and indistinguishable from knockout controls. Thus, bitter-receptor expressing cells mediate responses to bitter but not to sweet or amino acid tastants. These results illustrate a basic feature of taste coding: bitter is independently

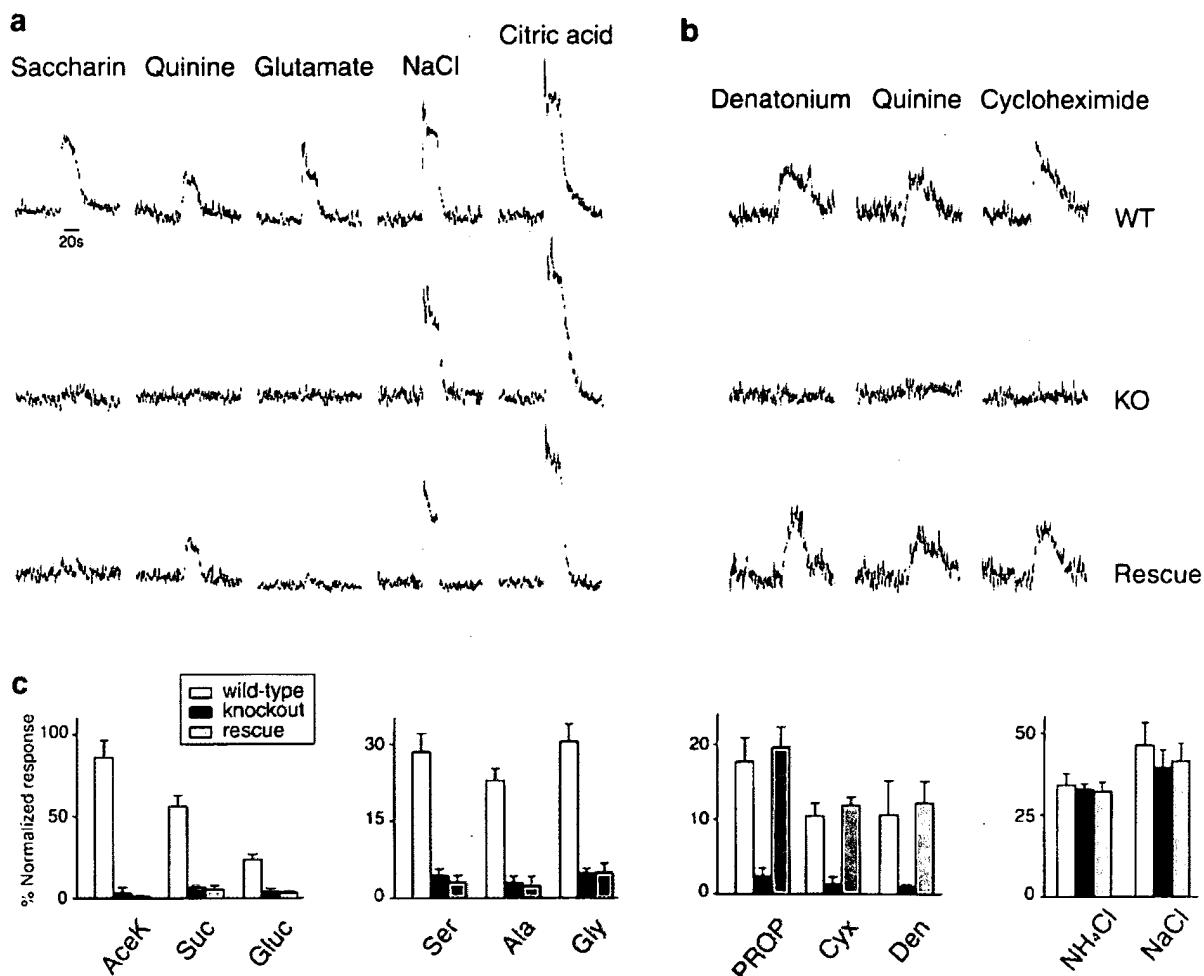


Figure 6. PLC β 2 Is Essential for Sweet, Amino Acid, and Bitter Taste Responses

(A) shows integrated chorda tympani responses to prototypical sweet, bitter, amino acid, salty, and sour tastants in wild-type (WT) and PLC β 2 knockout (KO). PLC β 2 knockouts have a complete loss of sweet, amino acid, and bitter taste responses. This phenotype mimics that of TRPM5 mutants. Expression of PLC β 2 in bitter receptor cells of PLC knockout mice (rescue) restores normal bitter but not sweet or amino acid responses.

(B and C) show responses to an expanded panel of tastants, both in glossopharyngeal (B) and chorda tympani (C) recordings. White bars, wild-type; red bars, PLC β 2 knockout animals; gray bars, bitter cell rescue animals. The values are means \pm SEM ($n = 4$) for normalized chorda tympani responses. Similar results were obtained for other stimuli (see Experimental Procedures).

encoded at the periphery and taste receptor cells are not broadly tuned across all taste modalities.

Concluding Remarks

To date, multiple signaling pathways have been proposed to mediate sweet, amino acid, and bitter taste reception. Surprisingly, even within a modality, a multitude of pathways have been hypothesized to activate taste receptor cells (see for example, Margolskee 2002). Sweet, amino acid (umami), and bitter taste reception are mediated by GPCR signaling via T1R and T2R taste receptors (Adler et al., 2000; Chandrashekhar et al., 2000; Nelson et al., 2001, 2002; Li et al., 2002). In this manuscript, we demonstrate that PLC, a major signaling effector of GPCRs, and TRPM5, a novel TRP related to the transduction channel of several sensory signaling processes, are co-expressed with T1Rs and T2Rs and are vital for sweet, amino acid, and bitter taste transduc-

tion. Our results showed that responses to all sweet (natural and artificial), amino acid (D- and L-), and bitter stimuli require these two signaling molecules. We propose that sweet, amino acid (umami), and bitter taste converge on a common transduction channel via PLC. In this model, tastant activation of T1R or T2R receptors would stimulate G proteins, and in turn PLC β 2. The activation of PLC, either directly or indirectly would lead to the gating of TRPM5 and the generation of a depolarizing receptor potential (Minke, 2001; Runnels et al., 2002). As in other sensory systems (Tsunoda et al., 1997; L'Etioile and Bargmann, 2000; Munger et al., 2001), additional pathways may modulate sweet, amino acid, or bitter taste reception but would not, themselves, trigger a taste response. Future experiments should help define the nature of the G proteins for the various taste modalities (Wong et al., 1996), and the mechanism whereby TRPM5 is gated.

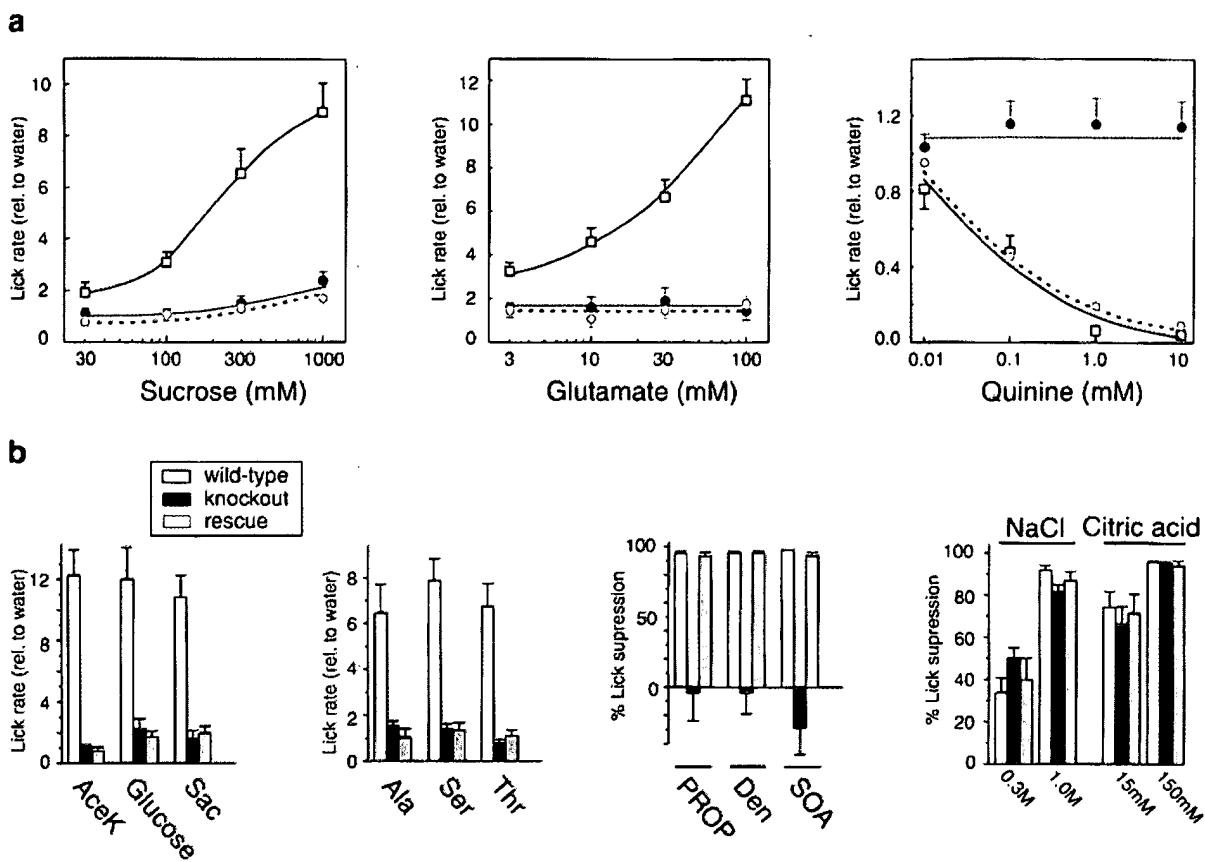


Figure 7. Targeted Rescue of Bitter Taste

Taste preference of wild-type (open squares, white bars), PLC β 2 (red circles, red bars), and rescue mice (gray circles, gray bars) were measured relative to water using a brief access taste test. PLC β 2 knockout animals exhibit a profound reduction in preference for sweet and amino acid tastants, a complete loss of bitter induced aversion but normal salty and sour responses. Expression of PLC β 2 in bitter receptor cells completely restores both the concentration dependence (A) and range (B) of bitter taste aversion but does not rescue sweet or amino acid taste. Note that responses to salty and sour stimuli are normal in the PLC β 2 knockout animals and are unaffected in the bitter-rescued animals. The values shown are means \pm SEM ($n = 8$). Sweet tastants: 32 mM acesulfame K, 2.5 M glucose, 32 mM saccharin; amino acids: 1 M alanine and serine, 300 mM threonine; bitter: 10 mM PROP and denatonium, 1 mM sucrose octaacetate (SOA).

Implications for Taste Coding

In this manuscript, we showed that animals lacking all sweet, amino acid, and bitter reception and perception still respond to sour and salty stimuli. These results formally demonstrate that sour and salty tastes are detected independently of other modalities. Two distinct models could explain these results: (1) separate populations of cells are uniquely tuned to these ionic tastes, or (2) they operate in the same cells that mediate sweet, amino acid, or bitter tastes, but there is no crosstalk between the modalities. Targeted ablation of selective populations of receptor cells may distinguish between these two possibilities.

Sweet, amino acid, and bitter receptors are expressed in distinct populations of taste cells (Hoon et al., 1999; Adler et al., 2000; Chandrashekar et al., 2000; Nelson et al., 2001, 2002). By successfully generating transgenic animals where only the bitter receptor-expressing cells possessed an active signal transduction pathway, we demonstrated that aversive responses do not require a functioning attractive (sweet and amino acid) pathway. Most significantly, these results strongly support our

hypothesis that there is functional segregation of taste modalities at the receptor cell level and question the soundness of current models of taste perception proposing that individual, broadly tuned taste cells detect sweet, amino acid, and bitter taste qualities. Deciphering the logic of taste coding will help us relate reception to perception and understand how the brain interprets gustatory stimuli. In the end, it is just a matter of taste.

Experimental Procedures

Molecular Cloning of TRPM5

We used a strategy that combined subtraction cloning and differential screening to isolate genes specifically expressed in taste receptor cells. In essence, a subtracted cDNA library from rat circumvallate papillae (Clontech PCR-Select cDNA Subtraction Kit) was probed with a collection of cDNAs derived from heart, brain, kidney, and liver to identify cDNAs expressed abundantly outside taste. Clones that did not hybridize were sequenced and assayed for taste specific expression by *in situ* hybridization. TRPM5 was isolated as a cDNA selectively expressed in a subset of taste receptor cells. Full-length clones were isolated as described previously (Hoon et al., 1999).

In Situ Hybridization and Immunostaining

Fresh frozen sections (16 μm /section) were attached to silanized slides and prepared for immunohistochemistry or *in situ* hybridization as described previously (Hoon et al., 1999). *In situ* hybridization was carried out using digoxigenin labeled probes at high stringency (hybridization, 5 \times SSC, 50% formamide, 65–72°C; washing, 0.2 \times SSC, 72°C). For fluorescent detection, horseradish-peroxidase conjugated goat anti-digoxigenin antibody was used in combination with tyramide-Cy3 (Perkin Elmer) to detect hybridization signal.

Anti-peptide antibodies to TRPM5 (against residues 1140–1156) were generated in rabbits and purified as described by Cassill et al. (1991). Anti-TRPM5 was detected with an Alexa 488-conjugated goat anti-rabbit secondary antibody (Molecular Probes) and images were obtained using a Leica SP2 TSC confocal microscope; 1–2 μm optical sections were recorded to ensure that any overlapping signal originated from single cells. For double-label experiments, *in situ* hybridization was carried out before immunohistochemical detection.

Gene Targeting of TRPM5

The strategy used to create TRPM5 knockout animals is shown in Figure 3. In the knockout, exons 15 to 19 encoding the first five transmembrane domains of TRPM5 were replaced by the PGK-neo' cassette. Homologous recombination was detected by diagnostic Southern hybridization of EcoRI + Xhol digested ES cell DNA with probes outside the targeting construct (see Figure 3). Two independently targeted ES clones were injected into C57BL/6 blastocysts. Chimeric mice were bred with C57BL/6 mice and progeny back-crossed to C57BL/6 mice for two generations; heterozygous animals were then crossed to generate homozygous knockouts. TRPM5 knockouts have normal viability, body weight, overall anatomy, and general behavior. Similarly, taste receptor cells appear normal morphologically and numerically in TRPM5 KO animals; they also express normal levels of taste receptors and other signaling molecules.

Transgenic Rescue of PLC β 2 Knockout Mice

Generation of PLC β 2-knockout mice has been described before (Jiang et al., 1997). PLC β 2 knockout animals were backcrossed with SWR mice for 5 generations prior to establishing a knockout line for testing and rescue. To generate the rescue construct, a 10 kb genomic fragment upstream of mouse T2R5 (Adler et al., 2000) was fused to PLC β 2 cDNA. A bovine growth hormone polyadenylation signal was used to allow detection of transgene expression by *in situ* hybridization. Transgenic lines were produced by pronuclear injection of zygotes from CB6 (BALB/c \times C57BL/6 hybrids) and were crossed into the knockout background. Three transgenic lines were tested for taste behavior and shown to restore aversive (bitter) but not attractive responses (sweet). No expression outside T2R-expressing cells was detected (Mueller et al.; our unpublished data).

Nerve Recording

Lingual stimulation and recording procedures were performed as previously described (Dahl et al., 1997; Nelson et al., 2002). Neural signals were amplified (5,000 \times) with a Grass P511 AC amplifier (Astro-Med), digitized with a Digidata 1200B A/D converter (Axon Instruments), and integrated (rms voltage) with a time constant of 0.5 s. Taste stimuli were presented at a constant flow rate of 4 ml min $^{-1}$ for 20 s intervals interspersed by 2 min rinses between presentations. All data analyses used the integrated response over a 25 s period immediately after the application of the stimulus. Each experimental series consisted of the application of six tastants bracketed by presentations of 0.1 M citric acid to ensure the stability of the recording. The mean response to 0.1 M citric acid was used to normalize responses to each experimental series.

Tastants used for nerve recordings were: sucrose, glucose, fructose, lactose, and sorbitol (300 mM); sodium saccharin and acesulfame K (10, 30, 60 mM); dulcin (5 mM); sodium cyclamate (60 mM); denatonium, 6-n-propyl thiouracil (10 mM); phenyl thiocarbamide (15 mM); cycloheximide (1 mM); quinine (0.1, 1, and 10 mM); salicin (80 mM); L-Glu, L-Ser, L-Ala, L-Asn, and L-Pro (30 mM with 0.5 mM IMP added); Gly, D-Ala, D-Phe, D-His, and D-Asn (100 mM). Amiloride (100 μM) was added to reduce sodium responses associated with these tastants for saccharin, cyclamate, and glutamate.

Behavioral Assays

Two-bottle preference assays were carried out as described previously (Nelson et al., 2001). For brief access tests, the lick rate of individual mice presented with series of different tastants was measured using a Davis MS160-Mouse gustometer (DiLog Instruments, Tallahassee, FL). Mice were trained and tested essentially as described by Glendinning et al. (2002). For behavioral testing, individual mice were placed in the gustometer for 30 min. Stimuli were presented in random order for 5 s trials that were initiated by the mouse licking the stimulus spout. In order to measure responses to attractive stimuli, mice were fed 1 g food and 2.5 ml water over the 24 hr before testing and were tested every second day. For aversive stimuli, mice were water deprived and were tested on a daily basis. To ensure adequate sampling, a maximum of 5 different solutions (including a water control) were presented during a test. For series of sodium saccharin and glutamate, 100 μM amiloride was added to all solutions to minimize effects of salt taste. Data points represent the mean rate that mice licked a tastant relative to their sampling of water for 4 animals tested twice with each series of stimuli; lick suppression is defined as 1 minus the lick rate relative to water.

Mammalian Cell Electrophysiology

Rat TRPM5 cDNA was subcloned into pEAK10 mammalian expression vector (Edge Biosystems, MD). Modified HEK-293 cells (pEAK^{rapid} cells; Edge BioSystems, MD) with or without G α 15 (Chandrashekhar et al., 2000) were seeded onto 35 mm culture dishes. After 24 hr at 37°C, cells were transfected using MIRUS TransIT-293 reagent (Mirus Corporation). Recordings were performed 48–72 hr after transfection. Bath medium was changed to Hanks balanced salt solution (GIBCO) containing 1 mM sodium pyruvate and 10 mM HEPES, 10–30 min prior to beginning of experiments. Electrophysiological recordings in the whole-cell patch clamp configuration were performed using AXOPATCH-1d amplifier and the pCLAMP 7 software (Axon Instruments). The recording pipette included 20 μM Fluo-3 (Molecular Probes) for measuring [Ca $^{2+}$]i changes and (in mM): KCl 140, MgSO $_4$ 2, Na-HEPES 10, ATP-Na $_2$ 2, and [pH 7.2]. Calcium imaging and UV-uncaging were performed using the LSM 510 confocal system mounted on an Axioscop 2 microscope (Zeiss). Fluo-3 fluorescence was monitored by excitation at 488 nm with a 505 nm long-pass emission filter; UV uncaging was achieved using 351 and 364 nm illumination. BAPTA, Caged InsP $_3$, and NP-EGTA (caged calcium) were purchased from Molecular Probes.

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